Depression of Weak Allograft Immunity in the Mouse by Neonatal or Adult Exposure to Urethan

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SUMMARY

Treatment of adult mice of three strains with minimally carcinogenic doses of urethan caused an impairment of cellular immunity as measured by prolongation of allograft survival. This effect was only detectable at the highest dose of urethan tested (150 mg/mouse) and was confined to the weaker strain combinations examined for allograft prolongation. Newborns appeared to be more susceptible to the depressive effect of urethan than did adults. In the BALB/c strain, doses of 120 and 150 mg urethan administered to adults were ineffectual in prolonging allograft survival. However, a dose of 1.0 mg administered to 4-day-old males (but not females) significantly prolonged allograft survival in some individuals when the mice were tested at 3 months of age. These facts are discussed in light of the pronounced carcinogenic effect of urethan in young and newborn mice.

INTRODUCTION

The fact that treatment of mice with hydrocarbon carcinogens routinely leads to the successful development of neoplastic cells with specific antigenicity led Prehn and Main (22) to propose that these carcinogens might facilitate tumorigenesis by interfering with the immune elimination of incipient tumors. The validity of this concept has been reinforced by the demonstration of the immunodepressive capability of hydrocarbon carcinogens (20, 26, 29, 31). The depression produced by hydrocarbons like 3-methylcholanthrene appears to be long lasting (1, 26, 31) and to include interference with both humoral (6, 26, 29, 31) and cellular (20, 26) immunity. The relevance of the depression has been demonstrated by Prehn (20) and Stjernsward (30), who have shown that minimally carcinogenic doses of 3-methylcholanthrene facilitate the outgrowth of small inocula of known antigenic tumors.

It is likely that the depressive effect of hydrocarbon carcinogens on cellular immunity is more important than their effect on humoral immunity in the development of nonviral tumors. The average latency of chemically induced tumors coincides with the onset of carcinogen-induced depression of cellular, rather than humoral, immunity (14, 29), and depression of cellular immunity as measured by allograft survival correlates highly with both antigenicity (2) and incidence (10) of chemically induced tumors. In general, the correlation between the carcinogenic activity of a given chemical with its depressive effect on cellular immunity is better than with its depressive effect on humoral immunity (26). Consequently, we have confined our study to an examination of the effect of a carcinogen on cellular immunity.

It has been suggested that the nonhydrocarbon carcinogen, ethyl carbamate (urethan), is an exception to the general pattern of antigenic tumorigenesis and coincident immune depression characteristic of hydrocarbon carcinogens (19). In the mouse, urethan-induced lung adenomas appear to be largely nonantigenic (19) and urethan has not as yet been shown to depress cellular immunity. Doses of urethan which interfere with humoral immunity (cf., Refs. 3, 15, 17) lack depressive effects on cellular immunity as measured by strongly incompatible (H-2 differing) tumor allografts (26). In 2 studies which demonstrated that urethan could potentiate the general immunodepressive effect of X-radiation (5, 9), there were no data to indicate a suppressive effect of urethan alone on cellular immunity.

To account for these facts within the general context of immunodepression and chemical carcinogenesis, we have tested the hypothesis that initiating and carcinogenic doses of urethan do have depressive effects on cellular immunity but that this effect is quantitatively weaker than that of the hydrocarbon carcinogens. In the 1st series of tests, we examined the relative strength of the depression produced by typical carcinogenic doses of urethan, and in the 2nd series we studied the duration of this depression by treating neonates with urethan and testing them as adults.

MATERIALS AND METHODS

The possibility that urethan has a weak immunosuppressive effect on cellular immunity was anticipated by designing allograft test systems in which there were 3 different degrees of incompatibility. The 1st test series was conducted in 4- to 6-month-old adults and consisted of the following groups: I, isografts of C57BL/6 male skin to C57BL/6 females; II, allografts of (BALB/c ×
DBA/2)F₁ hybrid skin to DBA/2 females; and III, allografts of DBA/2 skin to BALB/c females.

The strain combinations are arranged in increasing order of strength of antigenic differences. In Group I, the incompatibility between graft and host is weak and is due to the presence of the Y antigen in the male skin. In Groups II and III, where the donors and recipients share the same strong histocompatibility allele (H-2k), the incompatibility between graft and host is due to antigenic differences determined by alleles at multiple non-H-2 loci. The relative strengths of these differences are greater in the DBA/2 to BALB/c direction (Group III) than in the (BALB/c × DBA/2)F₁ to DBA/2 direction (Group II) (20).

One test series in each of the 3 groups received 150 mg urethan. In Group I and Group III, the urethan was administered to the graft recipients in 6 i.p. injections of 25 mg each (0.25 ml of a 10% solution) spaced 3 days apart. In Group II, the 6 injections were administered s.c. and were spaced 1 day apart. As a control for the anesthetic effect of the urethan injections, 0.25 ml of a 0.5% solution of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.) was administered to parallel control mice each time the experimental mice received a urethan injection.

In Groups I and III, additional test series which received smaller amounts of urethan were included. In Group I, a test series of graft recipients was given a single i.p. injection of 30 mg urethan (0.3 ml of a 10% solution). In Group III, a test series of graft recipients was given 4 weekly i.p. injections of 30 mg urethan. Simultaneous controls were treated as described above. These protocols approximate standard procedures and dosages used in specific studies where the carcinogenic or initiating effects of urethan were being studied (23, 24). Treated mice were test grafted as described below within 1 week of their last injection.

The 2nd series of tests was designed to examine the duration of the depressive effect of urethan. In view of the increased susceptibility of newborn mice to the carcinogenic action of urethan (25, 32), this series of tests was conducted in a strain of mice susceptible to lung adenoma formation (BALB/c) to determine whether neonatal exposure to a carcinogenic dose of urethan resulted in a long-lived depression similar to that induced by hydrocarbon carcinogens (1). This test series consisted of the following groups: IV, allografts of DBA/2 skin to BALB/c females; V, allografts of DBA/2 skin to BALB/c males; and VI, allografts of DBA/2 skin to thymectomized BALB/c males.

All experimental mice were treated when 4 days old with 1.0 mg urethan. In Groups IV and V, mice were anesthetized by cooling on ice and were given i.p. injections via a 28-gauge needle with 0.1 ml of a freshly prepared 1% solution of urethan in 0.85% NaCl solution. All control mice were given injections of 0.1 ml saline. In Group VI, thymectomies were performed within 48 hr of birth by the method of Sjödin et al. (27), followed 2 to 3 days later by urethan or saline treatment as described above.

A portion of the urethan- and saline-treated mice in Groups IV and V were treated when 10 weeks old with a sub-threshold dose of ALS¹ to determine if neonatal urethan treatment potentiated the immunodepressive effect of ALS. ALS was prepared by the method of Levey and Medawar (13) and 0.1 ml was injected i.p. for 5 consecutive days, followed 2 days later by 0.1 ml.⁵ Control mice received normal rabbit serum. Grafting was done on the 7th day following the inception of ALS or normal rabbit serum treatment.

All mice in Groups IV to VI were grafted when 11 to 12 weeks old. Grafts were made by the standard method of Billingham (4). In some tests, grafts were secured with Air-Vent tape (Johnson and Johnson, New Brunswick, N. J.) instead of plaster. Bandages were removed 8 to 10 days after grafting and the grafts were scored daily for evidence of rejection. Readings were done blind in that the cage cards in each group were not consulted until after the graft scores were made. The end point of rejection was considered as the day when 100% of the graft epithelium had been destroyed.

RESULTS

First Series. The results of the 1st test series, which include the MST's of each graft combination ±1 S.D. are shown in Table 1. Exact p values were calculated for each group from z values based on the Mann-Whitney U test (27). Since there were no significant differences in graft survival between the Nembutal control and control series in Groups I, II, and III, the data from both control groups were collated for statistical comparisons. Significant immune depression as measured by prolongation of allograft survival was obtained only in the weaker strain combinations tested (Group I and Group II), and only at the highest urethan dose tested (150 mg). In the weakest combination (Group I), this prolongation amounted to a doubling of graft survival (from 20.4 days in the Nembutal controls to 41.1 days in the series treated with 150 mg urethan). In the hybrid to parent combination (Group II), the prolongation of graft survival was more modest (from 17.2 days for the Nembutal controls to 20.5 days in the series treated with 150 mg urethan). There was no visible effect of urethan treatment on graft survival in Group III.

The prolongation of graft survival at the 150-mg dosage level was highly significant in Group I (p = 0.0073) and Group II (p = 0.003), but was not detected in Group III. In addition, there was a suggestive prolongation of graft survival at the 30-mg dosage level in Group I (p = 0.1058).

Second Series. The results of urethan treatment of the newborn are compiled in Table 2. In the strain combination tested, female BALB/c mice (Group IV) were im-

¹ The abbreviations used are: ALS, antilymphocyte serum; MST, median survival time.

² Unpublished work by M. A. L. established that a minimum of 0.25 ml/day ALS for 5 days was needed to effect immune depression in this test system.
Table 1
Graft survival times: mice treated as adults with urethan

<table>
<thead>
<tr>
<th>Group and strain combination</th>
<th>Controls</th>
<th>Nembutal controls</th>
<th>Urethan-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>MST ± S.D.</td>
<td>No.</td>
<td>MST ± S.D.</td>
</tr>
<tr>
<td>I.  C57BL/6♂ → C57BL/6♀</td>
<td>11</td>
<td>23.6 ± 1.7</td>
<td>12</td>
</tr>
<tr>
<td>II. (BALB/c × DBA/2)F1 → DBA/2♀</td>
<td>17</td>
<td>15.0 ± 1.1</td>
<td>15</td>
</tr>
<tr>
<td>III. DBA/2 → BALB/c♀</td>
<td>47</td>
<td>13.7 ± 1.1</td>
<td>14</td>
</tr>
</tbody>
</table>

Statistics

I. Controls and Nembutal controls vs. 30 mg urethan, $p = 0.1058$
Controls and Nembutal controls vs. 150 mg urethan, $p = 0.0073$

II. Nembutal controls vs. 150 mg urethan, $p = 0.0010$
Controls and Nembutal controls vs. 150 mg urethan, $p = 0.0003$

III. Controls and Nembutal controls vs. 120 mg urethan, n.s.
Controls and Nembutal controls vs. 150 mg urethan, n.s.

* n.s., not significant.

Table 2
Graft survival times: mice treated as newborns with 1 mg urethan

<table>
<thead>
<tr>
<th>Group and strain combination</th>
<th>Controls</th>
<th>ALS controls</th>
<th>Urethan</th>
<th>Urethan + ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>MST ± S.D.</td>
<td>No.</td>
<td>MST ± S.D.</td>
<td>No.</td>
</tr>
<tr>
<td>IV. DBA/2 → BALB/c♀</td>
<td>18</td>
<td>12.8 ± 1.1</td>
<td>8</td>
<td>11.0 ± 1.2</td>
</tr>
<tr>
<td>V. DBA/2 → BALB/c♂</td>
<td>24</td>
<td>14.0 ± 1.1</td>
<td>8</td>
<td>14.0 ± 1.1</td>
</tr>
<tr>
<td>VI. DBA/2 → BALB/c♂ thymectomized</td>
<td>16</td>
<td>14.7 ± 1.1</td>
<td>12</td>
<td>16.0 ± 1.1</td>
</tr>
</tbody>
</table>

Statistics

IV. Controls vs. ALS
Controls and ALS controls vs. urethan, n.s.
Controls and ALS controls vs. urethan and ALS, n.s.

V. Controls vs. ALS
Controls and ALS controls vs. urethan, n.s.
Controls and ALS controls vs. urethan and ALS, $p = 0.0097$
Urethan vs. urethan and ALS, n.s.

VI. Thymectomized controls vs. thymectomized, treated with urethan, $0.10 > p > 0.05$

* n.s., not significant

...munologically more reactive than males (Group V), as measured by MST of DBA/2 allografts (12.8 versus 14.0 days, respectively). As was seen in adult females tested in this combination (Group III), urethan treatment of newborn females with or without ALS was ineffectual in impairing adult immune reactivity. However, in BALB/c males (Group V), urethan treatment at 4 days resulted in a detectable disruption of the adult immune response. The distribution of MST's of controls and urethan-treated mice differed slightly (14.0 versus 14.8 days; $p = 0.1093$). A significant number of urethan-treated males showed allograft prolongation beyond the control limit of 17 days: [0/24 controls versus 6/22 urethan-treated, with MST's >17 days; $p < 0.025$ by Fisher test (7)]. The lack of statistical correlation between the test of distribution (U test) and the proportion test (Fisher test) is attributable to a spurious number of urethan-treated males exhibiting accelerated rejection (0/24 controls versus 3/22 urethan-treated males, with MST's <12 days). Experiments are under way to determine if this acceleration is a real phenomenon.

The suppressive effect of newborn treatment with urethan in males was more dramatically revealed by combining the urethan treatment with ALS. Whereas ALS alone had no discernible effect on allograft survival in males (both control and ALS-treated groups had MST's...
Depression of Allograft Immunity by Urethan

of 14.0 days), pretreatment with urethan followed by ALS produced a statistically highly significant prolongation of graft survival over control values (15.5 versus 14.0 days, respectively; \( p = 0.0087 \)). There was no significant difference between the MST's of the urethan and the urethan and ALS groups (14.8 versus 15.5 days, respectively; \( p > 0.2 \)).

Thymectomy alone in the BALB/c subline tested (BALB/cAnN1cr) does not result in a dramatic impairment of immunological reactivity over intact controls as measured by prolongation of allograft MST (14.7 versus 14.0 days, respectively; \( p > 0.2 \)). This is probably due to the presence of ectopic thymic tissue in this strain (11). Treatment of thymectomized BALB/c males with urethan significantly prolongs allograft MST when compared to intact controls (16.0 versus 14.0 days; \( p < 0.0003 \)). The prolongation in thymectomized males treated with urethan is of borderline significance when compared to corresponding thymectomized controls (16.0 versus 14.7 days; 0.10 > \( p > 0.05 \)). As seen before, more urethan-treated, thymectomized males had survival times over 17 days than did untreated controls (4/12 versus 1/16), but the numbers are too small to permit statistical evaluation. Taken together, however, these data strongly suggest that a proportion of BALB/c males, treated with urethan as newborns, have impaired allograft reactivity as adults.

**DISCUSSION**

These studies demonstrate that effective carcinogenic doses of urethan have a depressive effect on cellular immunity in the mouse as measured by prolongation of allograft survival. Newborns appear to be more susceptible to this depressive effect than adults, since in BALB/c mice, a dose of 1 mg/mouse given at 4 days of age results in greater immunodepression (Series II) than does 120 or 150 mg/mouse give at 3 to 5 months of age (Series I). It may prove that the pronounced susceptibility of newborn and weaning mice to the carcinogenic effect of urethan (25) is related to this age difference in immunodepressive effect.

The fact that in the adult immunodepression was only detectable at the highest dose of urethan used (150 mg) and in the weakest strain combinations tested suggests that the immunodepressive effect of urethan is quantitatively weaker than that of the hydrocarbon carcinogens which are effective in suppressing allograft immunity in graft systems with strong incompatibility (20, 26). This discrepancy may be attributable to the relatively rapid breakdown of urethan as compared to other carcinogens (16). However, the studies with mice treated as newborns (Series II) and those of Parmiani et al. (18) suggest that the immune depression produced by urethan, like that produced in newborns by the hydrocarbon carcinogens (1), is long lasting. Prolongation of immunodepression may be due to the damaging effect of urethan on the thymus (8). In the case of neonates, functional bone marrow injury may occur at critical stage in the development of the immune system and thereby impair thymus-dependent immune reactivity in later life.

The demonstration of the immunodepressive effect of urethan strongly reinforces the general validity of the correlation between immunodepression and carcinogenic activity previously established for the hydrocarbon carcinogens. In view of the demonstrated inverse relationship between degrees of immunological competence and strength of tumor antigenicity (2), the weakness of the depressive effect of urethan may in part be responsible for the relative weakness of the antigenicity of urethan-induced tumors (19).

Apparently, the degree of humoral depression is highly correlated with the leukemogeneic effect of urethan (18). The extent to which depression of cellular immunity is relevant to the other carcinogenic effects of urethan (e.g., lung adenoma formation) will be examined in another study.

**REFERENCES**


3. Berenbaum, M. C. Ectopic Thymic Tissue in This Strain (11). Treatment of thymectomized BALB/c males with urethan significantly prolongs allograft MST when compared to intact controls (16.0 versus 14.0 days; \( p < 0.0003 \)). The prolongation in thymectomized males treated with urethan is of borderline significance when compared to corresponding thymectomized controls (16.0 versus 14.7 days; 0.10 > \( p > 0.05 \)). As seen before, more urethan-treated, thymectomized males had survival times over 17 days than did untreated controls (4/12 versus 1/16), but the numbers are too small to permit statistical evaluation. Taken together, however, these data strongly suggest that a proportion of BALB/c males, treated with urethan as newborns, have impaired allograft reactivity as adults.


* Results of this study are now in press (11).


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